Receipt number	642-05-S-4467
Study number	44467

COMPANY SANITIZED DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

FINAL REPORT

Bioconcentration study of in carp

January 31, 2006

STATEMENT

Sponsor

Title Bioconcentration study in carp

Study number 44467

I, the undersigned, hereby declare that this report provides a correct English translation of the Final Report (Study No.44467, issued on January 31, 2006).

Date March 27, 2006

Study Director

GLP STATEMENT

Sponsor

Title Bioconcentration study carp

Study number 44467

This study was performed in compliance with :

(1) "Standard Concerning Testing Facility Relating to New Chemical Substances" (November 21, 2003; No. 1121003, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 17, 2003, No. 3, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121004, Environmental Policy Bureau, Ministry of the Environment)

(2) "OECD Principles of Good Laboratory Practice" (November 26, 1997)

This final report reflects the raw data accurately and it has been confirmed that the test data is valid.

Date January 31, 2006

Study Director Signed in original

QUALITY ASSURANCE STATEMENT

Sponsor

Title Bioconcentration study carp

Study number 44467

It has been assured that the final report accurately describes the test methods and procedures, and that the reported results accurately reflect the raw data of the study.

The inspections of this study were carried out and the results were reported to the Study Director and the Test Facility Management by Quality Assurance as follows.

Item of audit or inspection	Date of audit or inspection	Date of report to Study Director and Test Facility Management
Study plan draft	December 4, 2005	December 5, 2005
Study plan	December 5, 2005	December 5, 2005
Amendment to study plan	January 23, 2006	January 23, 2006
Acute toxicity test	December 6, 2005	December 8, 2005
Measurement of lipid content	December 13, 2005	December 14, 2005
	December 14, 2005	December 14, 2005
Preparation of stock solution	December 15, 2005	December 20, 2005
Analysis of test water	December 16, 2005	December 20, 2005
Analysis of test fish	December 19, 2005	December 20, 2005
Raw data and final report draft	January 31, 2006	January 31, 2006
Final report	January 31, 2006	January 31, 2006

Date January 31, 2006

Quality Assurance Unit, Head Signed in original

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Title

Bioconcentration study of carp

Sponsor

Test facility

Objective

This study was performed to evaluate the bioconcentration potential of carp.

Test method

This study was performed according to the following test methods.

(1) "Method for Testing the Degree of Accumulation of Chemical Substances in Fish Body" stipulated in the "Testing Methods for New Chemical Substances" (November 21, 2003, No.1121002, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 13, 2003, No.2, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No.031121002, Environmental Policy Bureau, Ministry of the Environment)

(2) "Bioconcentration : Flow-through Fish Test (Guideline 305, June 14, 1996)" in the OECD Guidelines for Testing of Chemicals

Applied GLP

This study complied with :

(1) "Standard Concerning Testing Facility Relating to New Chemical Substances" (November 21, 2003; No. 1121003, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare; November 17, 2003, No. 3, Manufacturing Industries Bureau, Ministry of Economy, Trade and Industry; No. 031121004, Environmental Policy Bureau, Ministry of the Environment)

(2) "OECD Principles of Good Laboratory Practice" (November 26, 1997)

Dates

Study initiation date	December 5, 2005
Experimental starting date	December 15, 2005
Experimental completion date	January 12, 2006
Study completion date	January 31, 2006

Storage of test item and raw data

(1) Test item

The item supplied by the sponsor is sealed in a storage vessel and stored in archives in this laboratory for ten years after the receipt of notice specified under Clause 1 or Clause 2 in Article 4, Clause 2 or Clause 3 or Clause 8 in Article 4-2, and Clause 2 in Article 5-4 or Clause 2 in Article 24 or Clause 2 in Article 25-3 of "Law Concerning Examination and Regulation of Manufacture, etc. of Chemical Substances". Treatment of the item supplied by the sponsor after the storage period is discussed with sponsor. If it is not stable for the storage period, it is stored as long while it is kept stable and it is disposed with approval of sponsor.

(2) Raw data and materials, etc.

Raw data, the study plan, documents concerning the study presented by the sponsor, the final report and necessary materials are stored in archives in this laboratory after the receipt of the notice specified under Clause 1 or Clause 2 in Article 4, Clause 2 or Clause 3 or Clause 8 in Article 4-2, and Clause 2 in Article 5-4 or Clause 2 in Article 24 or Clause 2 in Article 25-3 of "Law Concerning Examination and Regulation of Manufacture, etc. of Chemical Substances". Treatment of raw data and materials, etc. after the storage period is discussed with the sponsor.

Personnel

Study Director (2nd Chemical Safety Section)

Study personnel
(Operation of bioconcentration test)

Staff for fish care

Person to conduct of fish acute toxicity test

Approval of final report

Study Director Date January 31, 2006

Signature Signed in original

SUMMARY

Title

Bioconcentration study of : carp

Test conditions

Acute toxicity test

(1) Test fish	Orange-red killifish (*Oryzias latipes*)
(2) Duration of exposure	96 hours
(3) Exposure method	Semi static system (Renewal of test water, at every 24 hours)

Bioconcentration test

(1) Test fish	Carp (*Cyprinus carpio*)
(2) Nominal concentrations of test item	
	High exposure level (Level 1) 20 μg/L
	Low exposure level (Level 2) 2 μg/L
(3) Duration of exposure	28 days
(4) Exposure method	Continuous flow system
(5) Analytical method	Liquid chromatography-tandem mass spectrometry

Results

(1) 96-hour LC50 value	>500 mg/L
(2) Bioconcentration factors	Level 1 $\leqq 0.59$
	Level 2 $\leqq 5.8$

1. Test item

In this report, he following chemical name, etc.

1.1 Chemical name*1

1.2 Chemical structure, etc. *1

Structural formula

Molecular formula

Molecular weight

*1 Information supplied by the sponsor

2. Item supplied by the sponsor

2.1 Supplier and lot number*1

(1) Supplier

(2) Lot number RS4-56

2.2 Purity*1

(1) Test item 99.5 %(w/w)

(2) Impurity Water 0.5 %(w/w)

The test item was treated as 100 % in purity.

2.3 Confirmation of test item

Two infrared (IR) spectra of the test item provided by the sponsor and measured at this laboratory were confirmed to be identical (see Fig. 14 and Reference 2).

2.4 Physicochemical properties*1

Appearance	White solid
Melting point	
Stability	Stable at room temperature
	Stable to water, dimethylsulfoxide and acetone

*1 Information supplied by the sponsor

2.5 Storage and stability

(1) Storage condition

Dark storage place at room temperature

(2) Stability

The test item was stable under the storage condition as shown by the finding that IR spectra of the test item before and after the experiment were identical (see Fig. 14).

2.6 Stability under testing conditions

Prior to the bioconcentration test, a stability of the test item under the testing conditions was confirmed by a preliminary test.

3. Performance of acute toxicity test

3.1 Test method

The test was performed in accordance with Japanese Industrial Standard (JIS K 0102-1998-71.), "Testing methods for industrial waste water, Acute toxicity test with fish".

3.2 Test fish

(1) Species Orange-red killifish (*Oryzias latipes*)
Reason for selection : This species is similar in sensitivity to carp and readily available as test fish.

(2) Supplier Nakashima fish farm
(Address : 2029 Ooaza Nagasu Nagasu-cho Tamana-gun, Kumamoto 869-0123, Japan)

Date received July 28, 2005

(3) Conditions for fish care before acclimatization
Period

The fish were checked visually at receipt and those with any abnormalities were removed. The remainder was reared for 12 days in a flow-through system after the external disinfection for sick prevention and parasitic extermination.

External disinfection

The external disinfection for sick prevention was carried out in an aqueous solution containing 50 mg/L OTC (Oxytetracycline hydrochloride) for fisheries and 7 g/L sodium chloride for 24 hours. The external disinfection for parasitic extermination was carried out two times in an aqueous solution containing 30 µL/L formalin for 24 hours.

(4) Conditions of acclimatization
Period

After rearing, the fish were transferred to an acclimatizing aquarium and acclimatized there after the external disinfection. The fish showing any abnormalities during this period were removed and the remainder was reared for 43 days in a flow-through system at the temperature of 25 ± 2 °C. The fish were checked for health conditions and reared for 70 days after the external disinfection.

External disinfection

The first external disinfection was carried out in an aqueous solution containing 50 mg/L OTC for fisheries and 6 g/L sodium chloride for 24 hours. The second external disinfection was carried out in an aqueous solution containing 50 mg/L OTC for fisheries and 6 g/L sodium chloride for 24 hours.

(5) Weight average 0.23 g

(6) Length average 2.9 cm

(7) Certification

The 48-hour LC50 value of the reference substance*2 for the fish of the same lot (TFO-050812 II) was 0.642 mg/L.

*2 PCP-Na (pentachlorophenol sodium salt, Tokyo Kasei Kogyo Co., Ltd.)

3.3 Dilution water for test

(1) Origin

Groundwater from the premises of

(2) Water quality assessment

The dilution water for test was taken out on July 5, 2005, and it was analyzed and measured (once every six months in this laboratory). The results are shown in Reference 1.

It was confirmed that the dilution water met the requirements of at least one of the following standards.

① Ministerial ordinance of the Ministry of Health, Labour and Welfare No.101 (Revised May 30, 2003)
② OECD Guidelines for Testing of Chemicals, Fish, Early-life Stage Toxicity Test (Guideline 210, July 17, 1992)
③ Water quality criteria for fisheries (Japan Fisheries Resource Conservation Association, March 1983)
④ Environmental Quality Standards for Water Pollutants No.14 (Revised February 22, 1999, Environment Agency)
⑤ OECD Guidelines for Testing of Chemicals, Bioconcentration : Flow-through Fish Test (Guideline 305, June 14, 1996)

3.4 Preparation of stock solution

The test item was dissolved in ion-exchanged water to prepare 1000 mg/L stock solution.

3.5 Test conditions

(1) Test concentrations	500mg/L and Control
(2) Test tank	Round glass vessel
(3) Volume of test water	4 L / level
(4) Number of fish	10 / level
(5) Temperature of test water	
At initial exposure	24.5 °C
Before renewal of test water	24.0 - 24.1 °C
(6) Concentration of dissolved oxygen in test water	
At initial exposure	7.9 - 8.0 mg/L
Before renewal of test water	6.7 - 6.8 mg/L
(7) pH of test water	
At initial exposure	7.7 - 7.9
Before renewal of test water	7.7 - 7.9
(8) Duration of exposure	96 hours
(9) Exposure method	Semi static system (Renewal of test water, at every 24 hours)

3.6 Performance of test

(1) Place Aquatron room B

(2) Date December 5, 2005 - December 9, 2005

3.7 Estimation of 96-hour LC50 value

The 96-hour LC50 value was estimated by the Doudoroff method.

3.8 Result of test

96-hour LC50 value >500 mg/L (see Fig. 3)

4. Performance of bioconcentration test

4.1 Test fish

(1) Species Carp (*Cyprinus carpio*)
Reason for selection : The previous data conducted with this species can be compared and the size of this species is adequate for handling.

(2) Supplier Fukuoka fisheries and marine technology research center, freshwater fisheries laboratory
(Address : 2449 Yamada, Asakura-machi, Asakura-gun, Fukuoka 838-1306, Japan)

Date received	May 27, 2005
Starting date of acclimatization	October 26, 2005

(3) Conditions for acclimatization
Period

After rearing, the fish were transferred to an acclimatizing aquarium and acclimatized there after the external disinfection. The fish showing any abnormality during this period were removed and the remainder were reared for 28 days in a flow through system at the temperature of 25 ± 2 °C. The fish were checked for health conditions and transferred to test tanks. Thereafter the fish were reared at the same temperature in the flow through system for 20 days, following the external disinfection.

External disinfection

The external disinfection in the acclimatizing aquarium was carried out in an aqueous solution containing 50 mg/L OTC for fisheries and 7 g/L sodium chloride for 24 hours. The external disinfection in test tanks was carried out in an aqueous solution containing 50 mg/L OTC for fisheries and 7 g/L sodium chloride for 24 hours.

(5) Length 6.4 - 7.9 cm

(6) Lot No. TFC-051026

(7) Age Yearling fish

(8) Feeding

Feed	Feed for fry of carp
Composition	Proteins content ≧ 43.0 %
	Lipid content ≧ 3.0 %
Manufacturer	Nippon Formula Feed Mfg. Co., Ltd.

Feeding amount and interval

Amount corresponding to about 2 % of total body weight was fed twice a day in halves (once a day in all at holiday).

The fish were starved for 24 hours before sampling.

4.2 Dilution water for test

The same as described in Section 3.3.

4.3 Conditions of test and circumstances

(1) Supply of test water

Flow-through system assembled at this laboratory was used.

(2) Test tank

70-L glass tank

(3) Flow rate of test water

Level 1 and 2

2 mL/min for stock solution and 800 mL/min for dilution water, 1155 L/day of test water, were supplied.

Control

800 mL/min for dilution water, 1152 L/day of test water, were supplied.

(4) Stock solution bottle 25-L glass bottle
(Frequency of renewal 0 - 2 times/week)

(5) Temperature of test water

Level 1	24.0 - 24.4 °C
Level 2	23.9 - 24.2 °C
Control	24.2 - 24.5 °C

(6) Concentrations of dissolved oxygen in test water

Level 1	7.8 - 8.1 mg/L
Level 2	7.8 - 8.0 mg/L
Control	7.7 - 8.0 mg/L

(7) pH of test water

Level 1	8.1
Level 2	8.0 - 8.1
Control	7.9 - 8.2

(8) Time of irradiation with light

Artificial light of white fluorescent lamp (14 hours/day)

(9) Number of fish (at the beginning of exposure)

Level 1 and 2	29
Control	12

(10) Duration of exposure 28 days

Reason : A steady-state has been reached after 28 days.

(11) Place Aquatron room A

4.4 Preparation of stock solutions

- Level 1

1000 mg/L solution of test item was prepared in the same way as described in Section 3.4. 8 mg/L stock solution was then prepared from this solution by ionexchange.

- Level 2

1000 mg/L solution of test item was prepared in the same way as described in Section 3.4. 0.8 mg/L stock solution was then prepared from this solution by ionexchange.

4.5 Test concentrations

Based on preliminary test results for the 96-hour LC50 value and analytical detection limits, test concentrations of the test item were decided as follows. The control was set as a blank test.

Level 1	20 µg/L
Level 2	2 µg/L

4.6 Observation, measurement and cleaning of test tank

(1) Observation of test fish

Condition of test fish was observed visually twice a day (once a day at holiday).

(2) Flow rate of test water

Flow rate of stock solution and dilution water were measured with graduated cylinder and recorded once a day.

(3) Temperature of test water

Temperature of test water was measured with alcohol thermometer and recorded once a week.

(4) Concentration of dissolved oxygen in test water

Concentration of dissolved oxygen in test water was measured with dissolved oxygen probe and recorded once a week.

(5) pH of test water

pH of test water was measured with pH meter twice.

(6) Cleaning of test tank

In experimental period, excreta of carp, dirt on test tank, etc. were removed about once a day.

4.7 Analysis of test water and fish

Analysis of the test item in test water and test fish was performed with liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

4.7.1 Frequency of analysis

(1) Test water analysis

The test water of each level was analyzed once before first analysis of test fish and at the same time as analysis of test fish thereafter. The number of each sample was one.

(2) Test fish analysis

The test fish of Level 1 and 2 was analyzed five times in duration of exposure. Four fish were taken out at each sampling time and divided into two groups, then both were analyzed individually*3.

The control fish was analyzed before the experimental starting and after the experimental completion. Four fish were taken out at each sampling time and divided into two groups, and then each was analyzed individually. In addition, two fish were taken out and three groups (two fish per group) were used for measurement of lipid contents.

*3 Because one fish was too small to take out the stored sample for the measurement of lipid content, two fish a group were employed.

4.7.2 Pretreatment for analysis

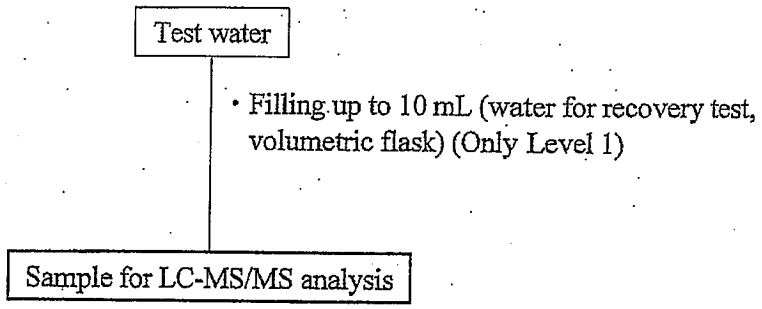
(1) Test water

An aliquot of the test water,

Level 1 1 mL

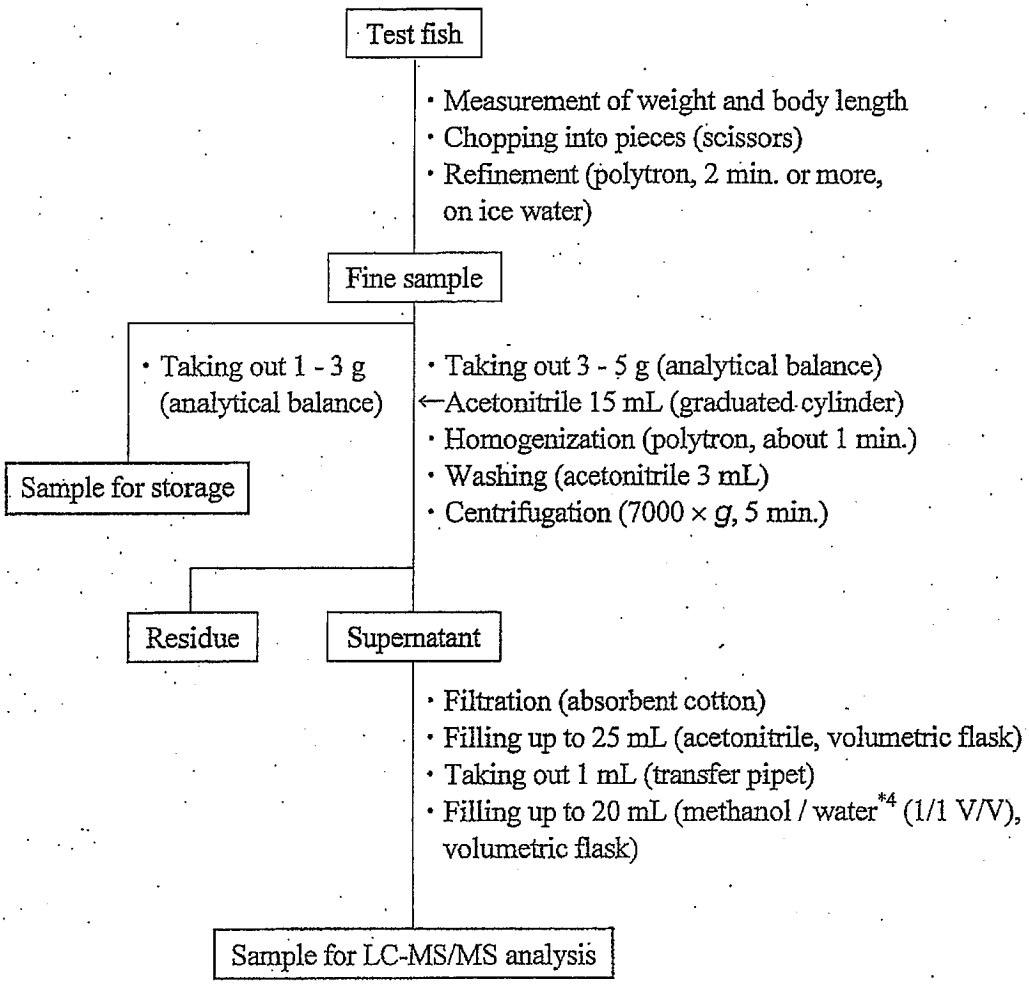
Level 2 10 mL

was taken from each test tank, and pretreated for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis as follows. However sample for analysis of Level 2 taken from test tank was a direct sample of LC-MS/MS analysis.



(2) Test fish

Test fish were taken from each test tank and pretreated for liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis as follows :



*4 City water was treated by Ultra pure water system.

4.7.3 Quantitative analysis for test item

The samples for LC-MS/MS analysis in pretreatment were analyzed by liquid chromatography-tandem mass spectrometry under the following analytical conditions. The concentration of the test item in each sample solution was determined on the basis of a comparison of the peak area on the chromatogram of the sample solution with that of a standard solution (see Tables-3, 4, Fig. 6 and Tables-6, 7, 8, Figs. 9, 10, 11).

(1) Analytical conditions

Instrument	Liquid chromatograph-mass spectrometer
Liquid chromatograph	Agilent type Agilent 1100
Mass spectrometer	Applied Biosystems/MDS Sciex type API4000

Conditions of liquid chromatograph

Column	L-column ODS (Chemicals Evaluation and Research Institute) 15 cm × 2.1 mm I.D.
Column temperature	40 °C
Eluent	A (30 %): Water[*4]
	B (70 %): Methanol[*5]
Flow rate	0.2 mL/min.
Injection volume	20 µL

Conditions of mass spectrometer

Ionization mode	Electrospray (ESI)
Detection ion	Negative
Detection mode	Selected reaction monitoring (SRM)
Precursor ion	
Product ion	
Turbo gas temp.	250 °C
Orifice plate voltage	-45.00 V
Collision energy	-10.00 V

*5 Containing 5mmol/L Di-n-butylammonium acetate

(2) Preparation of standard solution

The standard solution to determine the concentration of the test item in the sample solutions was prepared as follows.

(a) Test water

100 mg of the item supplied by the sponsor was accurately weighed and dissolved in water*4 to prepare 1000 mg/L solution of the test item. 2.00 µg/L standard solution was then prepared from this solution by dilution with water for recovery test.

(b) Test fish

100 mg of the item supplied by the sponsor was accurately weighed and dissolved in water*4 to prepare 1000 mg/L solution of the test item. 2.00 µg/L standard solution was then prepared from this solution by dilution with methanol / water*4 (1/1 V/V).

(3) Calibration curve

(a) Test water

1.00, 2.00 and 4.00 µg/L standard solutions were prepared by the same method as described in (2)(a). These solutions were analyzed according to the analytical conditions described in (1). A calibration curve was drawn on the basis of the relation between the peak area on the chromatograms and the respective concentrations.

The lowest detectable peak area of the test item was regarded as 45000 considering the noise level, which corresponded to the test item concentration of 0.10 µg/L (see Fig. 4).

(b) Test fish

1.00, 2.00 and 4.00 µg/L standard solutions were prepared by the same method as described in (2)(b). These solutions were analyzed according to the analytical conditions described in (1). A calibration curve was drawn on the basis of the relation between the peak area on the chromatograms and the respective concentrations.

The lowest detectable peak area of the test item was regarded as 45000 considering the noise level, which corresponded to the test item concentration of 0.10 µg/L (see Fig. 7).

4.7.4 Recovery and blank test

(1) Method

In the analysis of test water, recovery rate was employed as 100 % because pretreatment was only dilution. The blank test was conducted for test water (at water for recovery test) in the same manner without the test item. Fine sample of fish (10 g) spiked a specified amount of the test item for the recovery test was prepared in the same way as described in Section 4.7.2. The blank test was also performed in the same manner without the test item. 5 g of the fine sample was taken out at the recovery and blank tests for fish. All the recovery and blank tests were performed in duplicate.

(2) Results of recovery test

In the blank tests, the chromatogram of LC-MS/MS had no peaks interfering with determination of the test item concentration. The duplicate recovery rates and the average of them in the pretreatment are shown below (see Table-5 and Figs. 5, 8). The average recovery rate was used as correction factors for the determination of the test item concentrations in the analytical samples.

For analysis of test fish (2000 ng test item added)
91.3 %, 93.6 % average 92.4 %

4.7.5 Lipid content in test fish

Lipid contents in the sample for storage of the control test fish were determined with gravimetric analysis after chloroform-methanol extraction.

4.7.6 Calculation of the test item concentration in sample and minimum limit of determination

(1) Calculation of the test item concentration in test water

The equations in Tables-3 and 4 were used to obtain the concentrations, and they were rounded to 3 figures.

(2) Determination limit of the test item in test water

The determination limit*5 of the test item in test water was calculated on the basis of that obtained from the calibration curve in Section 4.7.3 (3)(a) as follows.

Level 1	1.0	μg/L
Level 2	0.10	μg/L

(3) Calculation of the test item concentration in test fish

If the test item was concentrated into the test fish, the equations in Tables-6, 7 and 8 were used to obtain the concentrations. However, the measured concentrations of test item were not more than the determination limit.

(4) Determination limit of the test item in test fish

Assuming the fine sample of fish to be 5 g, the determination limit*5 of the test item in test fish was calculated to be 11 ng/g on the basis of that obtained from the calibration curve in Section 4.7.3 (3)(b).

*5 Minimum determination limit of the test item (μg/L or ng/g)

$$= \frac{A}{\frac{B}{100} \times \frac{C \times E}{D}}$$

where

A : Minimum determination limit of the test item on the calibration curve (μg/L)

B : Recovery rate (%)

C : Sampling volume of test water (mL) or weight of fine sample of fish (g)

D : Final volume of sample solution (mL)

E : Ratio of the portion, used for analysis to whole volume

Results were rounded to 2 figures.

4.7.7 Calculation of average concentration of the test item in test water (duration of exposure)

$$\overline{Cwt} = \{ Cw(1) + \cdots\cdots + Cw(n) \} / n$$

where

$\overline{Cwt}$: The average concentration of the test item in test water (μg/L)
n : Number of analysis for test water (measurement times)
Cw(1): Concentration of the test item in 1st analysis of test water (μg/L)
Cw(n): Concentration of the test item in n-th analysis of test water (μg/L)

4.7.8 Calculation of bioconcentration factor (BCF)

Bioconcentration factor (BCF) was calculated as follows.

(1) Calculation of average concentration of the test item in test water for calculating BCF

$$\overline{Cw} = \{ Cw(n-1) + Cw(n) \} / 2 \quad \text{(only 1st analysis of test fish)}$$
$$\overline{Cw} = \{ Cw(n-2) + Cw(n-1) + Cw(n) \} / 3 \quad \text{(from 2nd analysis of test fish)}$$

where

$\overline{Cw}$: The average concentration of the test item in test water for calculating BCF (μg/L)
Cw(n): Concentration of the test item in n-th analysis of test water (μg/L)

(2) Calculation of bioconcentration factor

$$BCF = \frac{Cf}{\overline{Cw}}$$

where

BCF : Bioconcentration factor
Cf : Concentration of the test item in test fish (subtract FB) (ng/g)
$\overline{Cw}$: The average concentration of the test item in test water for calculating BCF (μg/L)
FB : Average concentration of the test item or blank in the control test fish analyzed before and after the experiment (μg/g)

(3) The average bioconcentration factor in m-th analysis

$$BCFm = (BCFa + BCFb) / n$$

where

BCFm	:	The average bioconcentration factor in m-th analysis (number of individual or group 2 (a,b))
BCFa,b	:	Each bioconcentration factor in m-th analysis of test fish
n	:	Number of individual or group in m-th analysis of test fish

The date of the analysis for test fish containing value when were not more than calculable BCF, BCFm is not calculated.

4.7.9 Definition of steady-state

The steady-state of BCF is defined to reach when the variation of BCFs in three successive analyses at intervals of more than 48 hours is within ±20 %. When BCFs are less than 100, it is evaluated that a steady-state has been reached after 28 days even if the variation of BCFs are over ± 20 %.

Criterion of the steady-state was reached : V(m-2), V(m-1), V(m) ≦ 20 (%)

$$V(m\text{-}2) = \frac{\left| BCF(m\text{-}2) - \overline{BCF} \right|}{\overline{BCF}} \times 100$$

$$V(m\text{-}1) = \frac{\left| BCF(m\text{-}1) - \overline{BCF} \right|}{\overline{BCF}} \times 100$$

$$V(m) = \frac{\left| BCF(m) - \overline{BCF} \right|}{\overline{BCF}} \times 100$$

V(m-2), V(m-1), V(m)	:	Variation rate of bioconcentration factor (%)
BCF(m-2), BCF(m-1), BCF(m)	:	The average bioconcentration factor in m-2, m-1, m-th analysis of test fish
$\overline{BCF}$	:	{ BCF(m-2) + BCF(m-1) + BCF(m) } / 3

4.7.10 Calculable BCF

On the basis of the minimum determination limit of the test item in Section 4.7.6 (4), BCF can be obtained when BCF exceeds the following. The average concentration of the test item in test water obtained from all the analyzed sample was used to calculate the following calculable BCF.

Level 1	0.59
Level 2	5.8

4.7.11 Calculation of lipid content

Lipid contents were calculated with the following equation.

$$\text{Lipid content (\%)} = (T - T_0) / S \times 100$$

where

T_0 : Weight of vessel (g)

T : Weight of sample for gravimetric analysis (containing vessel) (g)

S : Weight of fine sample taken out for analysis of lipid content (g)

4.8 Treatment of numerical values

Values were rounded in accordance with JIS Z 8401:1999 rule B. The each value used for calculation was used without rounding on the way of the calculation.

The concentration values of the test item in test water and fish were rounded to 3 figures. BCFs values were rounded to 2 figures.

5. Factors possibly affecting accuracy

No adverse effects on the reliability of this test were noted.

6. Results

6.1 Concentration of the test item in test water

The measured concentrations of the test item in test water are shown in Table-1. Each concentration of the test item was maintained at more than 91 % of each nominated concentration. The variation of the concentrations of the test item was within ± 20 % of the average of the measured concentrations.

Table-1 Measured concentrations of the test item in test water

(Unit : μg/L)

Level	After 1 day	After 4 days	After 7 days	After 12 days	After 21 days	After 28 days	Average (Standard deviation)	Table	Fig.
1	18.6	18.4	19.0	19.2	20.0	18.8	19.0 (0.58)	3	6
2	1.89	1.82	2.07	1.90	2.01	1.92	1.94 (0.092)	4	

6.2 Bioconcentration factors

BCFs are shown in Table-2.

BCFs in Table-2 plotted against the duration of exposure are shown in Figs. 1 and 2.

BCFs of the test item were not more than 0.59 at Level 1 and not more than 5.8 at Level 2.

Table-2 BCFs

Level	After 4 days	After 7 days	After 12 days	After 21 days	After 28 days	Table	Fig.
1	≦0.59 ≦0.59	≦0.59 ≦0.59	≦0.59 ≦0.59	≦0.59 ≦0.59	≦0.59 ≦0.59	6	9
2	≦5.8 ≦5.8	≦5.8 ≦5.8	≦5.8 ≦5.8	≦5.8 ≦5.8	≦5.8 ≦5.8	7	10

6.3 BCFs at a steady-state (BCFss)

Because the test item in all test fish at last three successive analyses were not more than minimum determination limit of test item, BCFss was not calculated. However, because all BCFs were less than 100, it was evaluated that a steady-state was reached after 28 days.

6.4 Lipid content in test fish

The measured lipid contents in the test fish are shown as follows.

Before initiation of experiment	2.43 %
After termination of experiment	2.48 %

6.5 Results of test fish observation

No abnormality in behavior or appearance was noted.

7. Remarks

Instruments, apparatus and reagents, etc. for the test

(1) Instruments for fish care

Micro quantitative pump for supplying stock solution : Tokyo Rika Kikai Co., Ltd. type GMW-A

Instrument for measuring concentration of dissolved oxygen : Iijima Electronics Co., Ltd. type ID-100

pH meter : Toa Electronics Ltd. type HM-14P

(2) Instruments, apparatus and reagents

Instruments and apparatus

Liquid chromatograph -mass spectrometer : see page 16

Electronic analytical balance : Sartorius AG type CP324S
Metler type AB204-S
A&D type FA-2000

Infrared spectrophotometer : Shimadzu Corporation type IRPrestige-21

Homogenizer (polytron) : Kinematica type PT3100

Centrifuge : Hitachi Koki Co., Ltd. type CR21G

Reagents

Acetonitrile (HPLC grade) :	Wako Pure Chemical Industries, Ltd.
Methanol (HPLC grade) :	Wako Pure Chemical Industries, Ltd.
0.5mol/L Di-n-butylammonium acetate (for Ion-Pair chromatograph) :	Tokyo Kasei Kogyo Co., Ltd.

(3) Instruments, apparatus and reagents for gravimetric analysis of lipid content in test fish

Instruments and apparatus

Electronic analytical balance :	Sartorius type BP301S
	Metler type AB204-S
Rotary evaporator :	Tokyo Rika Kikai Co., Ltd. type N-1000K
Homogenizer (polytron) :	Kinematica type PT3100
Homogenizer (autocellmaster) :	Iuchiseieido Co., Ltd. type CM-200
Vacuum pump :	Sinku Kiko Co., Ltd. type DA-20D
	Sinku Kiko Co., Ltd. type DTC-21
Vacuum desiccator :	Iuchiseieido Co., Ltd. type VL

Reagents

Purified water :	Takasugi Pharmaceutical Co., Ltd.
Methanol (extra pure) :	Wako Pure Chemical Industries, Ltd.
Chloroform (guaranteed reagent) :	Wako Pure Chemical Industries, Ltd.
Anhydrous sodium sulfate (extra pure) :	Kanto Chemical Co., Inc.

Table-3 Calculation table for analysis of test water

(Level 1)

Study No. 44467

Sample description	A	I
Standard 2.00μg/L	846998	
Test water after 1 day	786529	18.6
Standard 2.00μg/L	882542	
Test water after 4 days	812488	18.4
Standard 2.00μg/L	893476	
Test water after 7 days	849095	19.0
Standard 2.00μg/L	882389	
Test water after 12 days	845500	19.2
Standard 2.00μg/L	833488	
Test water after 21 days	834782	20.0
Standard 2.00μg/L	876917	
Test water after 28 days	822169	18.8

Average concentration of test item in test water 19.0 (S.D. 0.58)

A : Peak area

A(std) : Standard solution A(t) : Sample

B : Ratio of portion used for analysis 1

C : Final volume 10mL

H : Volume of test water taken out 1mL

I : Concentration of test item in test water (μg/L)

$I = P \times (A(t) / A(std)) / B \times C / H$

J : Average concentration of test item in test water (μg/L)

$J = (I(1) + ... + I(n)) / n$

n : Number of test water analyses (n = 6)

I (1) : First analysis of test water I (n) : Last analysis of test water

$$S.D. = \sqrt{\frac{n \times \sum_{i=1}^{n} I(i)^2 - \left(\sum_{i=1}^{n} I(i) \right)^2}{n \times (n-1)}}$$

P : Concentration of test item in standard solution 2.00μg/L

See Fig. 6

January 26, 2006

Table-4 Calculation table for analysis of test water
(Level 2)

Study No. 44467

Sample description	A	I
Standard 2.00μg/L	846998	
Test water after 1 day	799729	1.89
Standard 2.00μg/L	882542	
Test water after 4 days	801061	1.82
Standard 2.00μg/L	893476	
Test water after 7 days	925757	2.07
Standard 2.00μg/L	882389	
Test water after 12 days	838815	1.90
Standard 2.00μg/L	833488	
Test water after 21 days	838336	2.01
Standard 2.00μg/L	876917	
Test water after 28 days	843664	1.92

Average concentration of test item in test water 1.94 (S.D. 0.092)

A : Peak area

A(std) : Standard solution A(t) : Sample

B : Ratio of portion used for analysis 1

C : Final volume 10mL

H : Volume of test water taken out 10mL

I : Concentration of test item in test water (μg/L)

$I = P \times (A(t) / A(std)) / B \times C / H$

J : Average concentration of test item in test water (μg/L)

$J = (I(1) + \ldots + I(n)) / n$

n : Number of test water analyses (n = 6)

I (1) : First analysis of test water I (n) : Last analysis of test water

$$S.D. = \sqrt{\frac{n \times \sum_{i=1}^{n} I(i)^2 - \left(\sum_{i=1}^{n} I(i) \right)^2}{n \times (n-1)}}$$

P : Concentration of test item in standard solution 2.00μg/L

See Fig. 6

January 26, 2006

Table-5 Calculation table for recovery and blank test
(analysis of test fish)

Study No. 44467

Sample description	A	C	D	E	F	G
Standard 2.00μg/L	876896					
Recovery a	800357	1/25	20	-	1830	91.3
Recovery b	820626	1/25	20	-	1870	93.6
						Average 92.4
Standard 2.00μg/L	871256					
Blank a	n.d.	1/25	20	-	-	
Blank b	n.d.	1/25	20	-	-	
				Average -		

(a, b : individual sample)

A : Peak area

A(std) : Standard solution A(t) : Sample

B : Ratio of portion used for analysis (fine sample) 5/10

C : Ratio of portion used for analysis (extracted solution)

D : Final volume (mL)

E : Amount of blank in test fish (ng)

F : Amount of test item recovered (ng)

$F = P \times (A(t) / A(std)) / B / C \times D - E$

G : Recovery rate (%)

$G = F / Q \times 100$

P : Concentration of test item in standard solution 2.00μg/L

Q : Amount of test item added (2000ng)

See Fig. 8

January 16, 2006

Table-6 Calculation table for analysis of test fish
(Level 1)

Study No. 44467

Sample description	A	D	G	K	H	J	M
Standard 2.00μg/L	839498						
Test fish after 4 days a	n.d.	1	4.00	-	18.5	-	-
Test fish after 4 days b	n.d.	1	4.00	-	18.5	-	
Standard 2.00μg/L	873254						
Test fish after 7 days a	n.d.	1	4.00	-	18.7	-	-
Test fish after 7 days b	n.d.	1	4.00	-	18.7	-	
Standard 2.00μg/L	914136						
Test fish after 12 days a	n.d.	1	4.00	-	18.9	-	-
Test fish after 12 days b	n.d.	1	4.00	-	18.9	-	
Standard 2.00μg/L	822625						
Test fish after 21 days a	n.d.	1	5.00	-	19.4	-	-
Test fish after 21 days b	n.d.	1	5.00	-	19.4	-	
Standard 2.00μg/L	856418						
Test fish after 28 days a	n.d.	1	5.00	-	19.3	-	-
Test fish after 28 days b	n.d.	1	5.00	-	19.3	-	

(a, b : individual sample)

A : Peak area
A(std) : Standard solution A(t) : Sample
B : Ratio of portion used for analysis 1/25
C : Final volume 20mL
D : Dilution factor
E : Average concentration of blank in analysis of control 0ng/g
F : Recovery rate 92.4%
G : Weight of fine sample (g)
K : Concentration of test item in test fish (ng/g)
$K = \{ P \times (A(t) / A(std)) / B \times D \times C / G - E \} / F \times 100$
H : Average concentration of test item in test water (μg/L)
$H = \{ I(n-2) + I(n-1) + I(n) \} / m$; n : Number of test water analyses ; m = 2 when n = 2, m = 3 when n ≧3
I : Concentration of test item in test water (μg/L)
J : BCF
$J = K / H$
M : Average value of BCF(a) and BCF(b)
$M = \{ BCF(a) + BCF(b) \} / 2$
P : Concentration of test item in standard solution 2.00μg/L
See Fig. 9

January 26, 2006

Table-7 Calculation table for analysis of test fish
(Level 2)

Study No. 44467

Sample description	A	D	G	K	H	J	M
Standard 2.00μg/L	868136						
Test fish after 4 days a	n.d.	1	4.00	-	1.85	-	-
Test fish after 4 days b	n.d.	1	4.00	-	1.85	-	
Standard 2.00μg/L	882131						
Test fish after 7 days a	n.d.	1	4.00	-	1.93	-	-
Test fish after 7 days b	n.d.	1	4.00	-	1.93	-	
Standard 2.00μg/L	924241						
Test fish after 12 days a	n.d.	1	4.00	-	1.93	-	-
Test fish after 12 days b	n.d.	1	4.00	-	1.93	-	
Standard 2.00μg/L	839345						
Test fish after 21 days a	n.d.	1	5.00	-	2.00	-	-
Test fish after 21 days b	n.d.	1	5.00	-	2.00	-	
Standard 2.00μg/L	860951						
Test fish after 28 days a	n.d.	1	5.00	-	1.95	-	-
Test fish after 28 days b	n.d.	1	5.00	-	1.95	-	

(a, b : individual sample)

A : Peak area
A(std) : Standard solution A(t) : Sample
B : Ratio of portion used for analysis 1/25
C : Final volume 20mL
D : Dilution factor
E : Average concentration of blank in analysis of control 0ng/g
F : Recovery rate 92.4%
G : Weight of fine sample (g)
K : Concentration of test item in test fish (ng/g)

$$K = \{ P \times (A(t) / A(std)) / B \times D \times C / G - E \} / F \times 100$$

H : Average concentration of test item in test water (μg/L)

$$H = \{ I(n-2) + I(n-1) + I(n) \} / m$$; n : Number of test water analyses ; m = 2 when n = 2, m = 3 when n ≧ 3

I : Concentration of test item in test water (μg/L)
J : BCF

$$J = K / H$$

M : Average value of BCF(a) and BCF(b)

$$M = \{ BCF(a) + BCF(b) \} / 2$$

P : Concentration of test item in standard solution 2.00μg/L
See Fig. 10

January 26, 2006

Table-8 Calculation table for analysis of test fish (Control)

Study No. 44467

Sample description	A	E	G	I
Standard 2.00μg/L	849034			
Before the experimental start a	n.d.	-	3.00	-
Before the experimental start b	n.d.	-	3.00	-
Standard 2.00μg/L	827362			
After the experimental completion a	n.d.	-	5.00	-
After the experimental completion b	n.d.	-	5.00	-
				Average
(a, b : individual sample)				-

A : Peak area

A(std) : Standard solution A(t) : Sample

B : Ratio of portion used for analysis 1/25

C : Final volume 20mL

E : Amount of blank in analysis of control (ng)

$E = P \times (A(t) / A(std)) / B \times C$

G : Weight of fine sample (g)

I : Concentration of blank in test fish (ng/g)

$I = E / G$

P : Concentration of test item in standard solution 2.00μg/L

See Fig. 11

January 31, 2006

Reference 1 Analytical results of dilution water (Underground water)

Sampling date July 5, 2005

Item	Unit	Measured value	Standard value	Detection limit
Total hardness (Ca, Mg)	mg/L	12.4	< 300 *1	0.1
Suspended solid	mg/L	< 1	< 20 *2	1
pH	—	8.0	6.5 ~ 8.5 *3	
Total organic carbon	mg/L	1.9	< 2 *2	0.1
Chemical oxygen demand	mg/L	2.2	< 5 *3	0.5
Residual chlorine	mg/L	< 0.02	< 0.02 *3	0.02
Ammonia nitrogen	mg/L	0.02	< 1 *3	0.01
Total cyan	mg/L	< 0.01	n.d. *3	0.01
Alkalinity	mg/L	95	—	1
Electric conductivity	μS/cm	262	—	
Organic phosphorus	mg/L	< 0.1	n.d. *3	0.1
Alkylmercury	mg/L	< 0.0005	n.d. *3	0.0005
Mercury	mg/L	< 0.0005	< 0.0005 *3	0.0005
Cadmium	mg/L	< 0.001	< 0.01 *3	0.001
Cr^{6+}	mg/L	< 0.01	< 0.05 *3	0.01
Lead	mg/L	< 0.005	< 0.1 *3	0.005
Arsenic	mg/L	0.003	< 0.05 *3	0.001
Iron	mg/L	0.03	< 1.0 *3	0.01
Copper	mg/L	< 0.005	< 0.005 *3	0.005
Cobalt	mg/L	< 0.001	< 0.001 *5	0.001
Manganese	mg/L	< 0.01	< 0.05 *1	0.01
Zinc	mg/L	< 0.005	< 1.0 *1	0.005
Aluminium	mg/L	0.005	< 0.2 *1	0.001
Nickel	mg/L	< 0.001	< 0.001 *5	0.001
Silver	mg/L	< 0.0001	< 0.0001 *5	0.0001
Organochlorine pesticides				
1,2-Dichloropropane	mg/L	< 0.0001	< 0.06 *4	0.0001
Chlorothalonil	mg/L	< 0.0001	< 0.04 *4	0.0001
Propyzamide	mg/L	< 0.0001	< 0.008 *4	0.0001
Chlornitrofen	mg/L	< 0.0001	< 0.0001 *1	0.0001
Simazine	mg/L	< 0.001	< 0.003 *4	0.001
Thiobencarb	mg/L	< 0.0001	< 0.02 *4	0.0001
Organophosphorous pesticides				
Diazinon	mg/L	< 0.0001	< 0.005 *4	0.0001
Isoxathion	mg/L	< 0.0001	< 0.008 *4	0.0001
Fenitrothion	mg/L	< 0.0001	< 0.003 *4	0.0001
EPN	mg/L	< 0.0001	< 0.006 *4	0.0001
Dichlorvos	mg/L	< 0.0001	< 0.01 *4	0.0001
Iprobenfos	mg/L	< 0.0001	< 0.008 *4	0.0001
PCB	mg/L	< 0.0005	n.d. *4	0.0005
Coliform bacteria count	—	n.d.	n.d. *1	
Fluorine compound	mg/L	1.3	< 1.5 *3	0.1
Anionic surfactant	mg/L	< 0.01	< 0.2 *1	0.01

*1 Ministerial ordinance of the Ministry of Health, Labour and Welfare No. 101 (Revised May 30, 2003)

*2 OECD Guidelines for Testing of Chemicals, Fish, Early-life Stage Toxicity Test (Guideline 210, July 17, 1992)

*3 Water quality criteria for fisheries (Shadanhozin Nihon Suisansigen Hogokyokai, March 1983)

*4 Environmental Quality Standards for Water Pollutants No. 14 (Revised February 22, 1999, Environment Agency)

*5 OECD Guidelines for Testing of Chemicals, Bioconcentration : Flow-through Fish Test (Guideline 305, June 14, 1996)

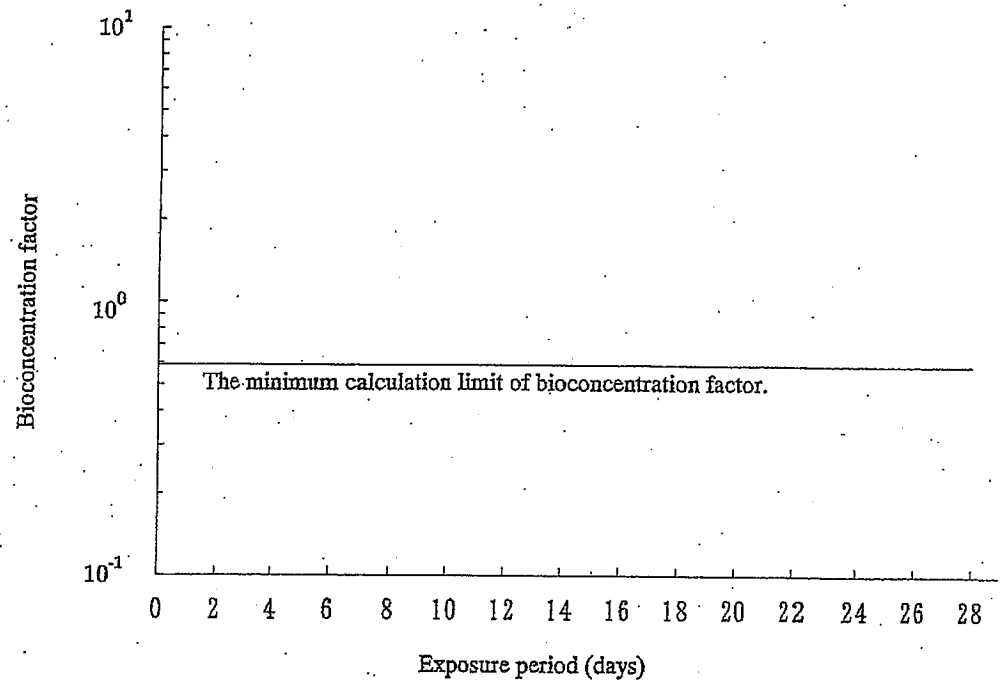


Fig. 1 Correlation between exposure period and bioconcentration factor (Level 1).
Ten data after 4, 7, 12, 21 and 28 days were lower than detection limit.

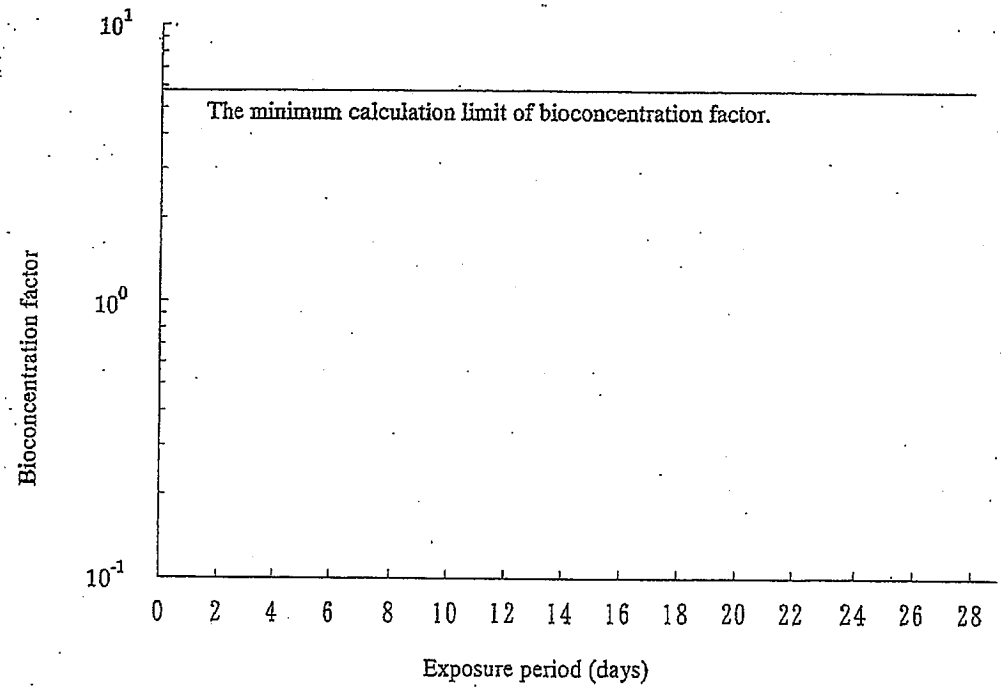
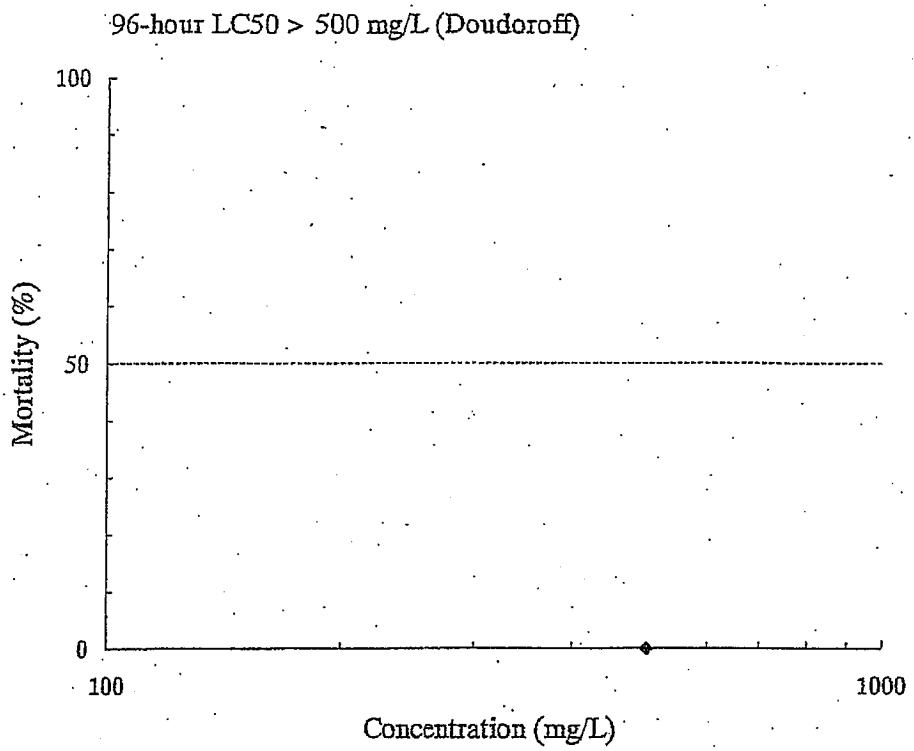


Fig. 2 Correlation between exposure period and bioconcentration factor (Level 2).
Ten data after 4, 7, 12, 21 and 28 days were lower than detection limit.



Concentration (mg/L)	Cumulative Mortality (%)			
	24 hours	48 hours	72 hours	96 hours
Control	0	0	0	0
500	0	0	0	0

Fig. 3 Concentration - mortality curve.

Date : December 9, 2005 Name

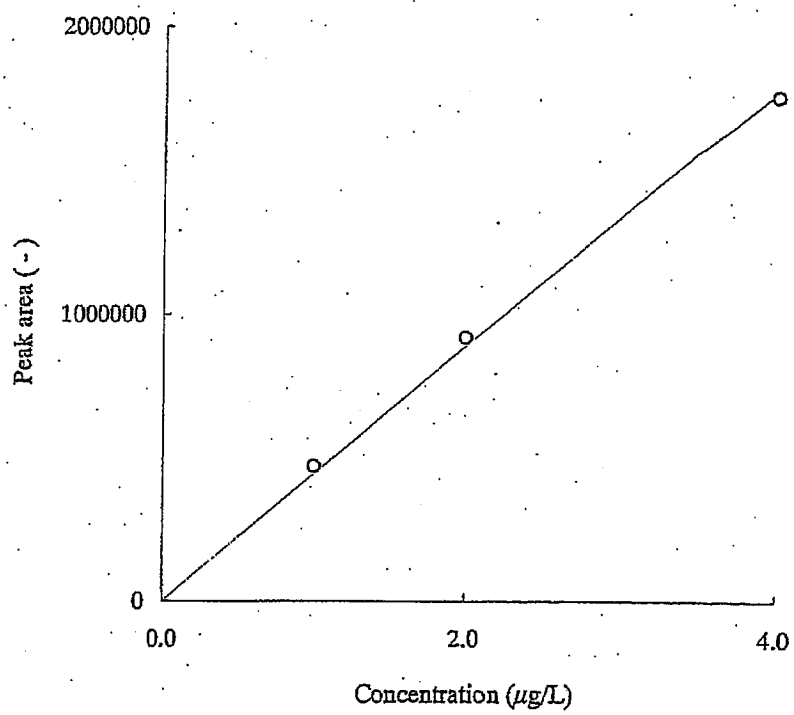


Fig. 4 - 2 Calibration curve of LC-MS/MS analysis for test item (test water).

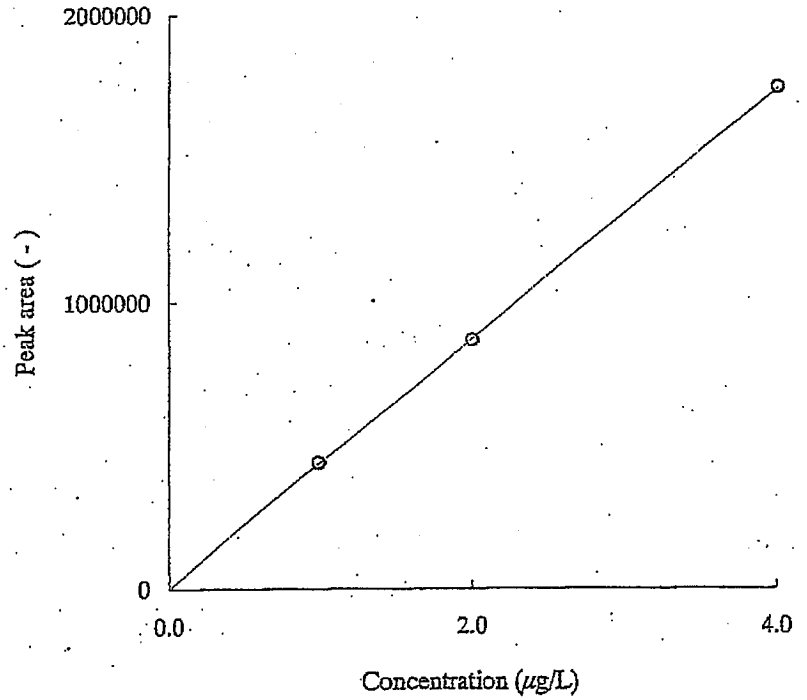


Fig. 7 - 2 Calibration curve of LC-MS/MS analysis for test item (test fish).

Instrument : Applied Biosystems/MDS Sciex API4000, Agilent 1100 Series

Sample : _____ mg/L

Solvent : Methanol / Water (1/1 V/V)

LC Conditions

Inlet system : Column

Injection vol. : 5 μL

Column (size) : L-column ODS (15cm × 2.1mm I.D.)

Column temperature : 40°C

Eluent : A (30%) : Water*

B (70%) : Methanol*

* containing 5mmol/L di-*n*-butylammonium acetate

Flow rate : 0.2 mL/min

MS Conditions

Ionization mode : ESI

Detection mode : Negative

Turbo gas temp. :

Orifice plate voltage : -45 V

Monitoring ion :

Fig. 12-1 Mass spectrum of test item.

Date 2005. 12. 6 Name Iwasaki

Instrument : Applied Biosystems/MDS Sciex API4000, Agilent 1100 Series

Sample :

Solvent : Methanol / Water (1/1 V/V)

LC Conditions

Inlet system : Column

Injection vol. : 5 μL

Column (size) : L-column ODS (15cm × 2.1mm I.D.)

Column temperature : 40°C

Eluent : A (30%) : Water*

B (70%) : Methanol*

* containing 5mmol/L di-*n*-butylammonium acetate

Flow rate : 0.2 mL/min

MS Conditions

Ionization mode : ESI

Detection mode : Negative

Turbo gas temp. : 250 °C

Orifice plate voltage : -45 V

Collision energy : -10 V

Precursor ion

Product ion

Fig. 13-1 Mass/Mass spectrum of test item.

Date 2005. 12. 6 Name Iwasaki